Four dimers of λ repressor bound to two suitably spaced pairs of λ operators form octamers and DNA loops over large distances

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Transcription factors that are bound specifically to DNA often interact with each other over thousands of base pairs [1,2]. Large DNA loops resulting from such interactions have been observed in *Escherichia coli* with the transcription factors deoR [3] and NtrC [4], but such interactions are not, as yet, well understood. We propose that unique protein complexes, that are not present in solution, may form specifically on DNA. Their uniqueness would make it possible for them to interact tightly and specifically with each other. We used the repressor and operators of coliphage λ to construct a model system in which to test our proposition. λ repressor is a dimer at physiological concentrations, but forms tetramers and octamers at a hundredfold higher concentration. We predict that two λ repressor dimers form a tetramer *in vitro* when bound to two λ operators spaced 24 bp apart and that two such tetramers interact to form an octamer. We examined, *in vitro*, relaxed circular plasmid DNA in which such operator pairs were separated by 2,850 bp and 2,470 bp. Of these molecules, 29% formed loops as seen by electron microscopy (EM). The loop increased the tightness of binding of λ repressor to λ operator. Consequently, repression of the λ PR promoter *in vivo* was increased fourfold by the presence of a second pair of λ operators, separated by a distance of 3,600 bp.

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Results and discussion

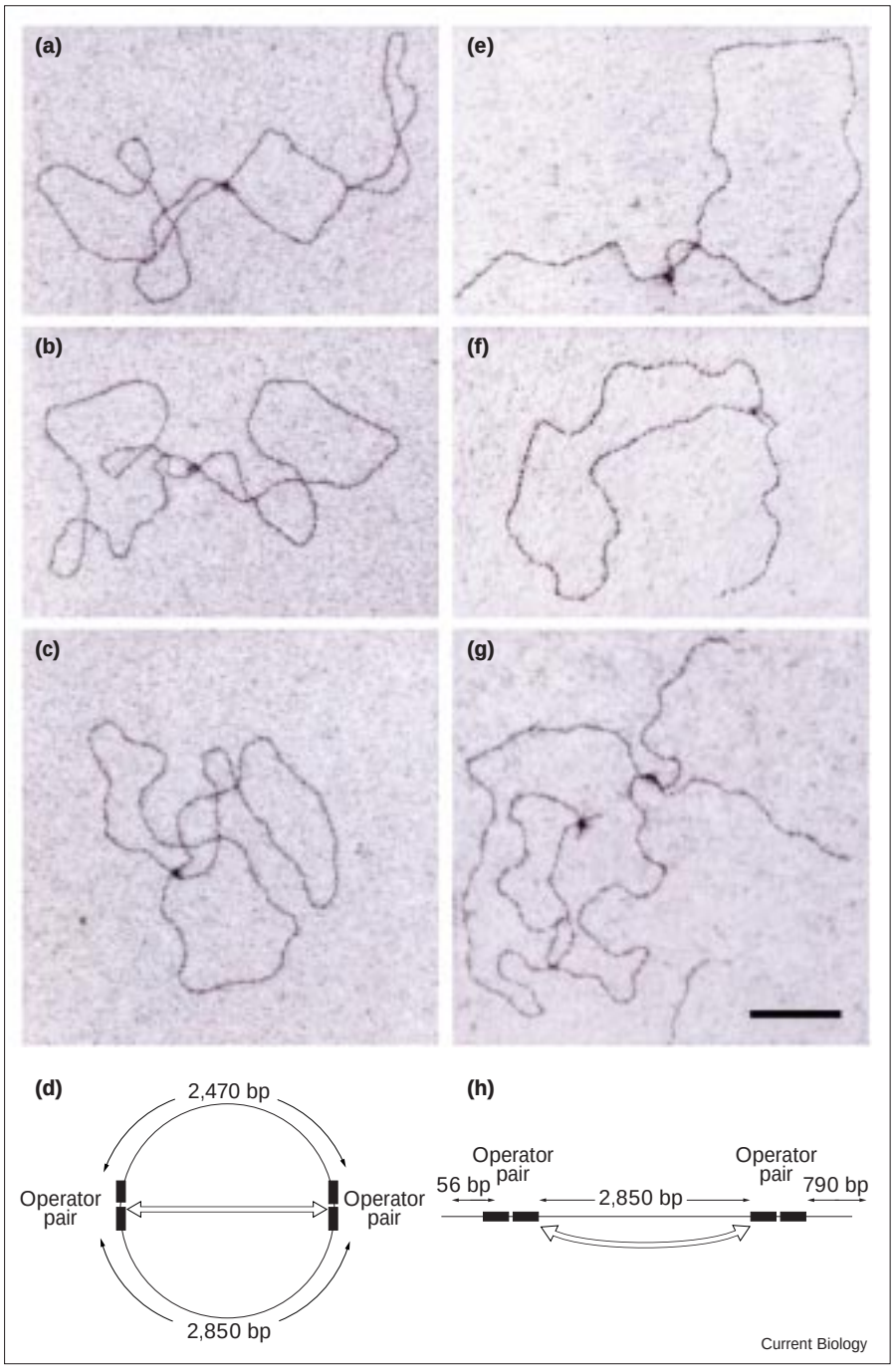
Theoretical background: the function of local concentration in short DNA–protein–DNA loops

In *E. coli*, the first DNA–protein–DNA loop was demonstrated in the arabinose operon system by Robert Schleif and colleagues in 1984 [5]; with a length of 211 bp, it belongs to the group of short loops. The best analysed system of this type is the *lac* system of *E. coli*, in which the function of the auxiliary operators 02 and 03, which are separated from the main operator 01 by 401 bp and 92 bp, respectively, is well understood [6]. When two subunits of a Lac repressor (LacR) tetramer bind to either 02 or 03, the remaining two subunits are brought close to operator 01. This raises the local concentration of LacR [6,7] in the vicinity of 01 by 15-fold or 5,000-fold (for 02 and 03, respectively) above the concentration of LacR in a wild-type *E. coli* cell (10^{-8} M). Local concentration effects are also observed in the formation of chelates and in the cyclisation of organic compounds. The influence of an auxiliary operator on the strength of repression of the *lac* promoter [8] thus decreases with increasing inter-operator distance and approaches zero at a distance of approximately 1,000 bp. The concentration of one LacR tetramer in a sphere with a radius of 1,000 bp equals the total concentration of LacR inside an *E. coli* cell — 10^{-8} M. Here, the chance of a tetrameric LacR molecule that is bound to one operator binding simultaneously to the other operator is not any greater than for any other LacR molecule in the cell. Most of the time these two operators will thus be occupied independently by two different tetramers. So, how can loops be formed over a distance of several thousand base pairs?

λ repressor is capable of forming loops over long distances

We propose that unique protein complexes, that are not present in solution, may form specifically on DNA. Their uniqueness makes it possible for them to interact tightly and specifically with each other. We used λ repressor, a paradigm of gene control [9], as a model. λ repressor is a dimer in solution at the wild-type intracellular concentration of 10^{-7} M [9]. Two dimers may form DNA loops between two operators separated by five or six turns of the DNA helix [10,11]. They bind cooperatively to two adjacent λ operators separated by 24 bp [9] by virtue of specific interactions between the carboxy-terminal, non-DNA-binding domains of the two dimers [12]. Free λ repressor forms octamers only at nonphysiological concentrations greater than 10^{-5} M [13,14]. It has recently been demonstrated that, at these high concentrations, λ repressor octamers bind specifically to two operators and non-specifically to adjacent DNA *in vitro* [15]. We predicted that λ repressor might readily octamerise at much lower concentrations when DNA is provided as a proper scaffold on which to preform tetramers by cooperative binding of two dimers to two adjacent operators. The two DNA-bound λ repressor tetramers may then interact with each

Figure 1



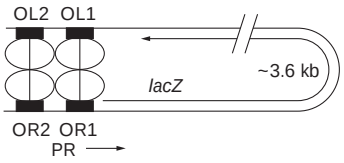
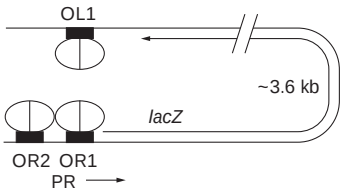
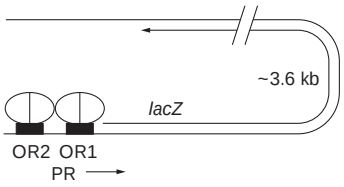
EM pictures of large DNA loops formed by λ repressor. (a–c) Circular relaxed plasmid DNA containing two pairs of operators at the wild-type distance of 24 bp between the centres of symmetry to allow the cooperative binding of λ repressor dimers to form tetramers. The operator pairs are separated by 2,850 and 2,470 bp respectively. The two tetramers on each DNA circle interact to form an octamer and thereby loop the DNA into a structure resembling a figure of eight. (d) The circular plasmid DNA used in (a–c). (e,f) DNA loops with linear DNA fragments excised from the plasmid shown in (d). The λ operator pairs are 2,850 bp apart. The centres of symmetry of the outer operators are located at distances of 56 bp and 790 bp from the ends of the molecule. (g) One of the rare sandwich structures in which two linear DNA molecules and two repressor octamers interact. The bar represents 0.1 μm. (h) The linear DNA used in (e–g).

other to form an octamer, irrespective of the length of the intervening DNA, because they are unique and the only tetramers present in most of the *E. coli* cells.

To study this possibility we analysed the interactions between λ tetramers bound to λ operators OL1–$O_{consensus}$, spaced 24 bp apart. We constructed a circular plasmid which carries two such pairs of λ OL1–$O_{consensus}$ operators separated by 2,850 bp and 2,470 bp (Figure 1d). We incubated either relaxed circular plasmid DNA or a purified linear restriction fragment with purified λ repressor at physiological concentrations and determined loop formation by EM (Figure 1). On the linear fragment, the operator pairs are located 56 bp and 790 bp from the respective ends and the distance between them is 2,850 bp (Figure 1h).

Figure 2

Increase of repression by operators positioned 3,600 bp downstream of the start of transcription. (a) Schematic representation of the control elements and their position with respect to the reporter gene (*lacZ*). PR is the right promoter region of bacteriophage λ, including operators OR1 and OR2. The presence of additional operators OL1 and OL2 downstream of *lacZ* is indicated by black boxes. Repressor dimers are represented by spheres. (b) Specific β-galactosidase activities, determined in the absence and presence of approximately wild-type amounts of λ repressor. (c) Repression values are calculated as: (specific β-galactosidase activity in the presence of λ repressor divided by the specific β-galactosidase activity in the absence of λ repressor)–1 in order to obtain a value of 0 for no repression.

(a) Proposed structure	(b) Specific activity of β-galactosidase in the absence of λ repressor	(b) Specific activity of β-galactosidase in the presence of λ repressor	(c) Repression
	180	3.5	50
	170	13	12
	180	13	13

Current Biology

When λ repressor (10^{-7} M per dimer) was allowed to interact with the relaxed circular plasmid DNA (10^{-9} M) carrying the pairs of λ operators, 58 of 200 molecules (29%) presented themselves in a characteristic figure of eight shape, with a λ repressor octamer forming the loop (Figure 1a–c). The fraction of the DNA molecule determined to be in the larger loop was 0.535 ± 0.006. This value agrees well with the value predicted from the construction of the plasmid (0.533). With linear fragments carrying the pairs of λ operators at a distance of 2,850 bp, we observed 200 molecules by EM of which 24 (12%) formed characteristic large loop structures (Figure 1e,f). Two sandwich structures [16], composed of two independent DNA molecules and connected by two λ repressor octamers, could also be identified (Figure 1g).

The λ repressor loop has a function in increasing repression

Does octamer formation stabilise the binding of each dimer to its operator? We used an indirect method, the *in vivo* measurement of repression, to address this question. We constructed three plasmids which carry the *lacZ* gene under the control of the λ PR promoter and OR1–OR2 and which differ only in the presence or absence of λ OL operators downstream of the *lacZ* gene (Figure 2). They are thus separated from the λ PR promoter by 3,600 bp. From these plasmids, fragments comprising the λ promoter, the *lacZ* gene, the downstream region and the *bla* gene (Figure 2a) were excised and inserted into the *E. coli* chromosome [17] of a strain carrying a *lac* deletion. The specific activities of β-galactosidase [18] were determined in all three strains in the presence and absence of approximately wild-type amounts of λ repressor (Figure 2). It can be seen that a fourfold increase in repression occurs only in the presence of two λ operators downstream of the *lacZ* gene. This suggests that loop formation also occurs *in vivo* and that the binding of the λ repressor dimers to their operators is tightened by octamer formation. It seems likely, therefore, that such an octamer is formed in the lysogenic state, stabilising the interactions of λ repressor with promoters PR and PL and so stabilising the lysogenic state. We therefore predict the existence of mutant λ repressors that are unable to form octamers but that are still able to form tetramers. Such lysogens should lyse more frequently than wild-type lysogens.

Protein assembly on DNA, a generalisation of the scaffold model

Can this model be generalised for eukaryotic enhancers, which often reside tens of thousands of base pairs upstream or downstream of their promoters? Indeed, this model has been alluded to by authors using terms like ‘recruitment’ [19] or ‘cross talk’ [20]. The principle — to use DNA as a scaffold on which to assemble proteins at specific positions and thereby to create a protein complex with a unique surface that would not assemble in liquid except at much higher concentrations — is presumably widely used in nature. Well-characterised examples exist

for two site-specific recombinases [21,22] and a recent study indicates that the phosphorylated form of the bacterial transcription activator NtrC may behave in a similar manner to that described here for λ repressor [23]. A similar mechanism may also be used in the process by which eukaryotic chromosomes align, because such long-distance interactions function in *trans* almost as well as in *cis*. Finally, DNA is indeed an ideal scaffold on which to create unique complexes. However, other macromolecules, such as the cytoskeleton or membrane, may also be used for this purpose.

Materials and methods

Plasmid construction and in vivo *measurements*

Double-stranded DNA for λ promoter regions or λ operators was synthesised on an Applied Biosystems 394 DNA/RNA synthesiser, gel purified and cloned into a pBR322 derivate upstream or downstream of a *lacZ* gene according to standard procedures [24]. We used the wild-type sequence of PR with OR1–OR2 and in OL1–OL2 we inactivated PL by suitable base substitutions. The relevant sequence of OL2–OL1 is: 5′-TATCTCTGGCGGTGTTGtagTAAATACCACTGGC-GGTGATAtaG (small letters indicate altered bases). From the resulting plasmids, fragments comprising the promoter, the reporter gene, the downstream control region and the β-lactamase gene were inserted into λ lP1 [17], which then was integrated into the chromosome of the *E. coli* strain BMH8117 $(lac–pro)_{\Delta}$ [25]. Single lysogens were purified and transformed with $pSX100C_I$ or $pWB\Delta C_I$ [26]. β-galactosidase activity was determined as described [18].

In vitro *experiments*

λ repressor was purified essentially as described [27] and stored at –70°C at a dimer concentration of 2×10^{-4} M. For complex formation, it was diluted 100-fold in binding buffer (10 mM HEPES/KOH, pH 7.5; 20 mM KCl; 0.1 mM EDTA; 0.1 mM DTT; 0.33 mM Mg acetate; 0.33 mM CaCl) in BSA-coated tubes, rinsed twice with binding buffer prior to use. 0.2 or 0.4 μl of the repressor dilution was added to 10 μl binding buffer containing 0.1 μg DNA. The concentration of the DNA was $2–5 \times 10^{-9}$ M, the concentration of λ repressor dimers was $4–8 \times 10^{-8}$ M. The sample was incubated on ice for 10 min. 2 μl of the binding reaction was further diluted 10-fold immediately prior to spreading onto pentylamine-activated carbon-coated grids (7 μl), which were washed and stained with 2% (w/v) uranyl acetate solution in water and dried on filter paper [28]. Note that our buffer conditions differ from those described by Pray *et al.* [15]. Our differing results might be explained by the apparent effect of the concentration of Mg^{2+} and Ca^{2+} ions on λ repressor octamer formation [29]. The grids were analysed by use of annular dark-field illumination mode with a Zeiss CEM-902 microscope. Images were recorded on Kodak electron image film (Eastman Kodak) at a magnification of 50,000×, 85,000× or 140,000×. The prints were inverted using an image scanner in order to improve the final printing. A Dage SIT camera fitted to the electron microscope allowed images to be directly analysed and digitised. Contour length measurements were performed with custom-designed image analysis programmes [30].

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